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Separation and Validation of Comprehensive Impurities in Erythromycin Tablets by using Rp-HPLC method

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Abstract
In this study a simple, rapid, accurate and precise gradientRP-HPLC method has been developed and validated for the separation of impurities (IMP) in Erythromycin (ERT-A) tablets pharmaceutical dosage form. The chromatographic separation was carried out on WatersX-Terra RP 18 (250 mm x 4.6 mm I.D., 3.5 µm particle size) at 65°C was used for this separation. Mobile Phase-A consists of Buffersolution (35 g of di-potassium hydrogen phosphate in 1000mL of water, adjust the pH7.0with dilute o-phosphoric acid, filtered through 0.45 µm membrane filter) acetonitrile and water in ratio of 5:35:60 v/v/v respectively. Mobile Phase-B consists of a mixture of phosphate buffer pH 7.0, water and acetonitrile in ratio of 5:45:50 v/v/v respectively .The flow rate and injection volume was 1.0 mL/min and 100 µL respectively. The analysis was carried out under the gradient conditions as time (min)/A (v/v): B(v/v); T0/100:00, T45/100:00, T47/0:100, T63/0:100, T65/100:00, and T70/100:00. The wavelength was identified at 215 nm. The ERT-Adegrades under various circumstances. The degradation products were well resolved from the ERT peaks. This method was found to be linear. The cumulative %RSD values are ;identified and found that they are within the range. From the study concluded that the method is accurate, specific, selective, precise, robust, and useful during the process development and quality check in finished dosage form manufacturing.
Keywords: *Erythromycin, HPLC, forced degradation, LOD,LOQ, Accuracy.*

1. Introduction
Erythromycin (ERY) is a therapeutical compound belonging to the class of macrolide antibiotics, originally discovered by McGuire et al.[1]the molecular formulae is $C_{37}H_{67}NO$and the molecular weight is 733.94 g/moland later produced through biosynthesis during fermentation from species of the Gram-positive Saccharopolysporaerythraea, formerly classified as Streptomyces erythraeus [2,3]. Erythromycin is useful for treating various infections and also has an indication for a non-infectious pathology. Traditionally, its use has been for various respiratory infections, prophylaxis of neonatal conjunctivitis, and chlamydia [4]. Santhosh Kumar Ettaboina et al.,[5] used the mobile phases A as Buffer (pH 9.0 Dibasic Potassium Phosphate), tertiary butyl alcohol, Acetonitrile in the ratio of 800:170:30 v/v/v), Mobilephase B is a purified Water and ; Mobilephase C is Acetonitrile. For this analysis authors are used Agilent PLRP-S, 250mm x 4.6 mm, 8µm, 1000A°. Wavelength is measured at 215 nm and authors discussed about topical formulation only. SalikaJeelani et al.,[6]proposed this method for separation of various impurities in this proposed drug by UHP-QDa with Column as Waters XBridge C18 (100 mm × 4.6 mm, 3.5 µm), maintained the wavelength at 215 nm. The mobile phase is 0.4 % ammonium hydroxide in water and methanol. Chirag J Patel et.al.,[7] proposed method to determine the low level of erythromycin. For this analysis used BEH C18, (50 mm x 2.1 mm, 1.7 µm) and 210 nm. B. Habibi et al.,[8] proposed with to sepration with an AsahipakShodex ODP-50 4E column (250 mm × 4.6

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mm i.d.,5 µm particles) column with HPLC, measurement was taken at Wavelength 210 nm and authors used this method to determination of erythromycin, clarithromycin, and azithromycin residues in fish muscles. FahimehKamarei et al.,[9] chromatographic separation was achieved on an X-Terra C18 analytical column UV detection at 205 nm for Assay of erythromycin only. Jacqueline Wardrop et al.,[10] A C18 Polymeric column is used with UV detection at 205 nm. During the study not discussed about Forced degardation studies. K. Griessmannet et al.,[11]Method is used at UV Detection 215 nm and Column: polymerX column (4.6 mm 25.0 cm, 7 µm). The method is only limited to determine Erythromycin without estimation of impurities. Lakshmana Rao A et al.[12] quantified Erythromycin tablet dosage forms was carried out on Hypersil BDS C18 column (150 mm x 4.6 mm I.D., 5 µm particle size) UV detection was performed at 224 nm, And proposed method doesn't have any forced degradation data and method is only for Assay of Erythromycin in Erythromycin tablet dosage forms. Zhiling Cao et al,[13] The chromatographic analysis was performed on Inertsil C18 ODS column at Wavelength of the UV detector was 215 nm. All four related substances, including Z isomer of erythromycin A oxime, erythromycin A, erythromycin A-6, 9-hemiketal and erythromycin A-6, 9-9, 12-spiroketal were separated and determined under isocratic elution, But Forced degradation study was not performed. A. S. Rathore et.al., [14], developed and validated method for quantification of Isotretinoin and Erythromycin in bulk drug and topical gel formulation The separation was carried out on Merck TLC aluminum sheets of silica gel 60 F254, (20 × 10 cm) with 250 µm thickness. Used this technique for TLC and not have studied about Erythromycin and its related impurities. Verified the USP Erythromycin Tablets and not found any related substance method for Erythromycin and Its related impurities[15]. The related substance method available in current USP monograph of Erythromycin API[16]. European pharmacopeia Erythromycin API monograph listed related impurities is verified for API but placebo peaks and blanks peaks was not separated[17].

To address these gaps in the existing literature, the objective of our study was to devise a straightforward and expeditious stability-indicating method tailored for the quantification of ERT (ERT) and its associated impurities in Tablets (OSD) formulations. By using literature reviews authors are carried out this method for the development of Erythromycin and it's associated impurities in Tablets (OSD) formulations. Further developed method was validated as per ICHQ(2A) guidelines as shown in (Figure 1).

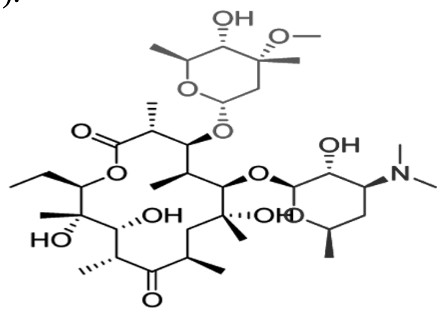

Figure 1 Chemical Structures of ERT
https://en.wikipedia.org/wiki/ERT

2. Materials and Methods

2.1. Standard and Impurities

ERT (93.9% purity), Anhydro ERT A (Imp d) (92.2% purity) (Fig 2), ERT A enol ether (Imp. E) (94.5% purity)(Fig 3), Pseudo ERT A enol ether (Imp. F) (91.2% purity) (Fig 4), ERT Imp. H (ERT A 3´´-N-oxide) (Purity 86.7%) (Fig 5)and ERT Imp. L (3"-N-demethyl-3"-N-formyl ERT A (89.7% purity) (Fig 6) were obtained from Dr Reddy's Laboratory, Hyderabad, India .ERT Related Compound N (Imp. B) (95.3 % purity) (Fig 7) were obtained from USP.

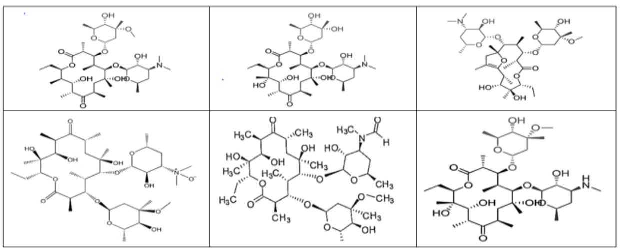

Figure 2 AnhydroERT A (Imp. D), ERT A enol ether (Imp. E), PseudoERT A enol ether (Imp. F), ERT A 3´´-N-oxide (Imp. H)ERTImp. L, ERT Related Compound N(Imp. B), (3"-N-demethyl-3"-N-formylERT A)

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2.2. Experimental

Authors obtained analytical/HPLC-grade tertiary butyl alcohol from Alfa Aesar. The monobasic potassium phosphate, dibasic potassium phosphate, and O-Phosphoric acid as analytical reagent (AR) grade and procured from Spectrum Chemicals, Mumbai, India. HPLC-grade methanol and acetonitrile were procured from J. T. Baker. Di-potassium hydrogen phosphate and orthophosphoric acid from Merck Life Sciences. Used high-quality purified water (Milli-Q) for all experimental analyses. The chromatographic analysis was conducted using a Waters Alliance 2695 Module from Waters Corporation in the USA. The HPLC system was composed of various components, including a quaternary gradient pump (QSM), an autosampler a column oven, and a PDA detector. The LC column used was the Waters X-Terra RP 18 with a particle size of 3.5 µm and dimensions of 4.6 mm x 250 mm, which was manufactured by Waters.

2.3. Optimization of Chromatographic

Conditions. Mobile Phase-A consists of a mixture of buffer solution, acetonitrile, and water as 5:35:60v/v/v. Mobile Phase-B consists of phosphate buffer at pH 7.0, water, and acetonitrile in a volumetric ratio of 5:45:50v/v/v. The flow rate is identified at 1.0 mL/min. Volume injected is 100 µL. Temperature was maintained at 65°C where as the sample temperature was maintained at 10°C. Wavelength detected at 210 nm. Run time was 70 minutes. The results obtained are represented in the table 1.

Table 1 Gradient Program

Time (min)	Mobile phase A (% v/v)	Mobile phase B (% v/v)
0	100	0
45	100	0
47	0	100
62	0	100
65	100	0
70	100	0

2.4. Preparation of solutions

For this analysis prepared various solutions includes diluent solution, diluted standard solution, resolution solution and retention time solution, sample solution, and Impurity spiked sample solution. By using the said solutions the following chromatograms are obtained and represented in the figure 3 reveals that the chromatograms of resolution and diluted standard and figure 4 represents impurities present in the ERT sample.

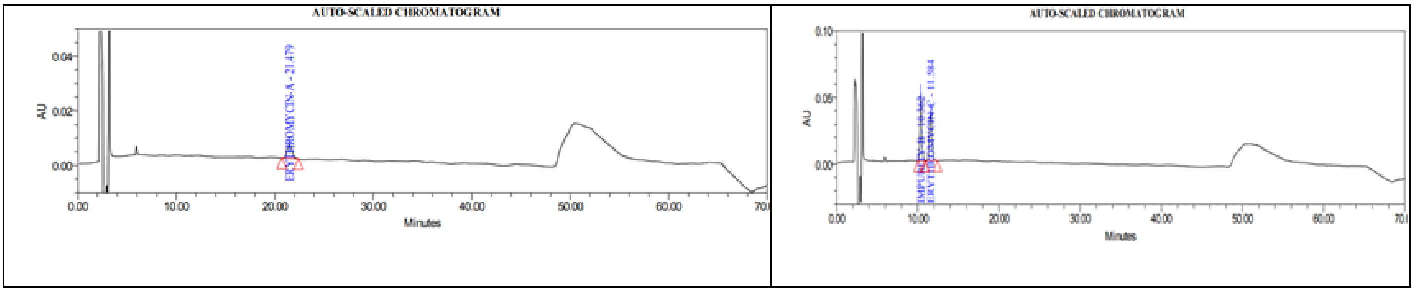

Figure 3 ERT standard and Resolution Solution Chromatograms

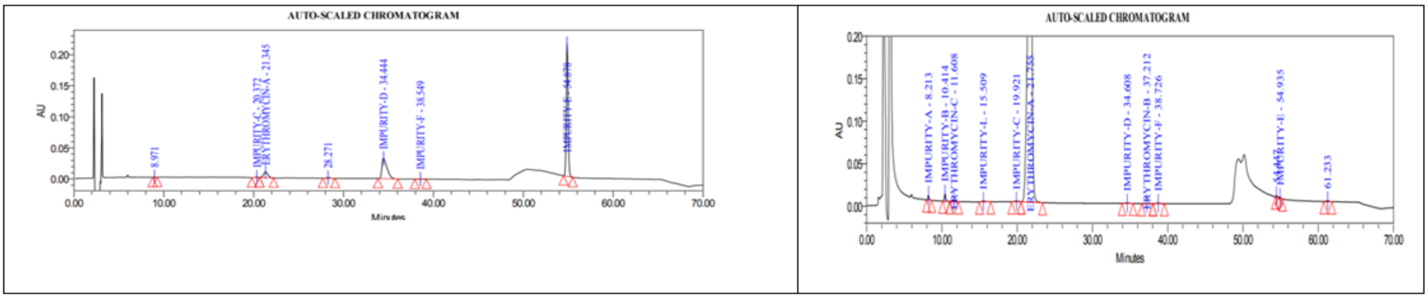

Figure 4 Chromatograms of ERT A enol ether (Imp. E) and Spiked Sample with Impurities

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3. Results and Discussion

3.1. System suitability

To assess the system's suitability, we began by sequentially injecting four distinct solutions into the HPLC system: a Blank solution (a single injection), the Standard solution, the Imp.-E Retention Time (RT) solution, and the Resolution solution. Each solution was carefully prepared. A comprehensive analysis of the system suitability results confirms that the method is indeed suitable and reliable for further evaluation. The detailed results can be found in table 2.

Table 2 System Suitability Results

System suitability parameters	Obtained Result
The resolution between ERT Related Compound N (Impu B) and ERT C in resolution solution.	2.4
Tailing factor from diluted standard chromatogram (First replicate injection of Standard)	1.2
The Relative standard deviation for replicate injections of diluted standard preparation.	0.4

3.2. LOQ and LOD

The limits of detection (LOD) and Limit of quantification (LOQ) were established at signal-to-noise ratios of 3:1 and 10:1, respectively, by introducing a series of diluted solutions containing known concentrations of the target components into a placebo matrix. To assess precision and accuracy, a study was conducted at the LOQ concentration. Six separate spiked preparations (n = 6) were accurately prepared, and the obtained results were found to consistently meet the validation criteria specified in Table 3.

Table 3 LOD and LOQ Results

Name of the Imp.	LOD (%)	LOQ (%)	LOQ	
			Precision (%RSD)	Accuracy
ERT A 3 N-oxide (Imp.-H)	0.013	0.034	2.9	97.5
ERT Related compound N (Imp.-B)	0.008	0.029	3.6	98.3
3-N-demethyl-3-N-fromyl ERT A(Imp.-L)	0.002	0.006	0.0	98.8
Anhydro ERT A (Imp.-D)	0.055	0.120	3.2	105.3
Pseudo ERT A enol ether(Imp.-F)	0.005	0.013	0.0	95.3
ERT A enol ether(Imp.-E)	0.001	0.003	0.0	102.4
ERT A	0.029	0.090	3.2	97.8

3.3. Linearity

The linearity of the method was established through the validation process by injecting solutions at six concentration levels, spanning from the Limit of Quantification (LOQ) to 150% of the Level of specification for each component of interest, including Impurities like H, B, L, D, F, E, and ERT A. This evaluation involved performing a least-squares linear regression analysis by plotting peak area against the concentration data, resulting in a linear regression coefficient exceeding 0.998. The Linearity graph for ERT A can be referred to as shown in Figure 5, and a summary of the results pertaining to the method's linearity is provided in Table 4.

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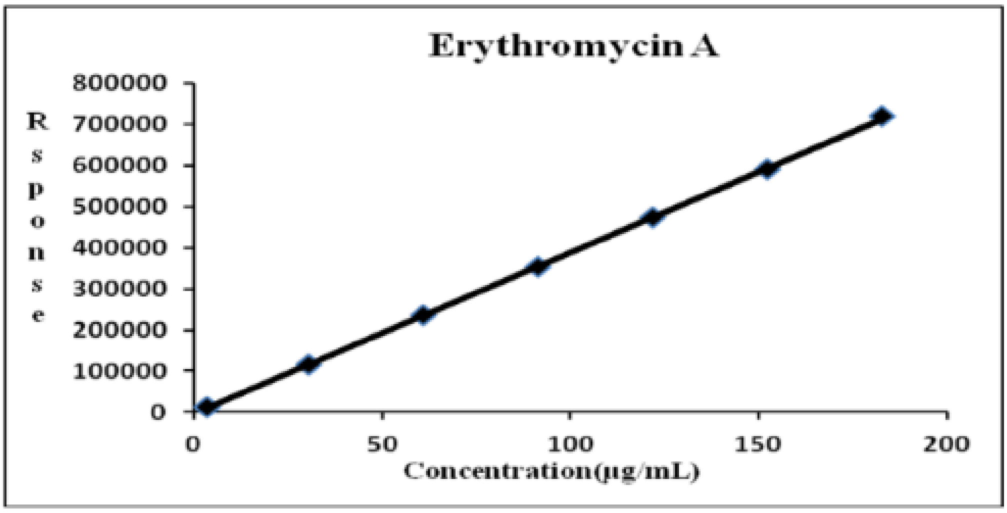

Figure 5 Linearity graph of ERT A

Table 4 Linearity Results

Parameter	Imp.-H	Imp.-B	Imp.-L	Imp.-D	Imp.-F	Imp.-E	ERT-A
Range(µg/mL)	1.176-59.5637	1.2096-119.9923	0.2373-24.6840	4.6696-58.4710	0.4837-62.5121	0.1193-180.3196	3.6077-182.7917
Correlation coefficient	0.999908	0.999916	0.999920	0.999328	0.999991	0.999959	0.999929
Intercept	690.8571	1375.1608	3816.1817	332.6382	6178.3838	14906.4015	3606.4752
Slope	3653.02050	3589.70139	45564.31996	1700.68122	41571.27261	48011.16791	3920.95129

3.4. Precision and Accuracy

The precision of the method was assessed by analyzing by preparing six ERT Tablets Formulation solution samples and spiking ERT related impurities (Impu H, Impu B, Impu L, Impu D, Impu F, Impu E) at the specification level of test concentration. The %RSD (Relative Standard Deviation) was calculated for all impurities. The intermediate precision study was conducted by different analysts on different days, using different systems and columns. %RSD values for all impurities were calculated, and the cumulative %RSD for both method precision and intermediate precision was determined. Similarly, method precision and intermediate precision were evaluated for an unknown Imp. (ERT A) spiked into a placebo. Accuracy testing involved the preparation of a sample of ERT Tablets solution by spiking impurities at levels ranging from the Limit of Quantification (LOQ) to 150% of the Imp. specification level in the test solution. Additionally, ERT A, an unknown Imp., was spiked into the placebo at the specification level. The % recovery values were found to be within the acceptable limits of 85.0% to 115.0%. The results of the method validation for precision, intermediate precision, and accuracy are summarized in Table 5.

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Table 5 Accuracy and Precision Results

Parameter	Imp.-H	Imp.-B	Imp.-L	Imp.-D	Imp.-F	Imp.-E	ERT-A
LOQ Accuracy (Mean, 95% Confidence low & High)	97.5,96.8 & 98.2	98.3, 88.8 & 107.8	98.8, 93.4 & 104.2	105.3, 104.0 & 107.2	95.3, 102.0 & 102.8	102.4, 94.5 & 96.1	97.8,95.5 & 100.1
50% Accuracy (Mean, 95% Confidence low & High))	97.9, 96.8 & 99.0	98.6, 97.6 &99.6	97.4, 95.9 & 98.9	96.5, 91.9 &94.7	100.7, 99.4 & 102.0	104.6, 102.5 & 106.7	100.9, 100.3 & 101.5
100% Accuracy (Mean, 95% Confidence low & High)	96.4, 93.5 & 99.3	101.2, 98.5 & 103.9	98.6, 95.6 & 101.6	102.8, 92.3 & 113.3	104.3, 102.9 & 105.7	102.7, 100.0 & 105.4	103.6, 101.8 & 105.4
150% Accuracy (Mean, 95% Confidence low & High)	99.2, 98.0 & 100.4	98.3, 97.1 & 99.5	97.8, 96.6 & 99.0	91.2, 90.0 & 92.4	100.9, 100.3 & 101.5	103.3, 102.1 & 104.5	105.5,104.9 & 106.1
Repeatability (%RSD)	1.1	1.4	1.4	6.9	0.8	1.0	1.0
Intermediate precision (%RSD)	3.0	1.5	3.0	4.2	0.4	0.9	0.3
Cumulative (%RSD)	5.1	4.0	3.8	5.8	2.8	4.3	3.1

3.5. Specificity

Impurities H, B, L, D, F, E, and ERT A are measured at their target levels. The resulting chromatogram was analyzed at the Rt of each component of interest. The results demonstrate that the proposed method is specific for the precise estimation of ERT and its related impurities in tablet formulations. Chromatograms for the placebo and impurities spiked into the test solution are provided in Figure 6 for the typical placebo chromatogram for the typical chromatogram of impurities spiked in the test solution.

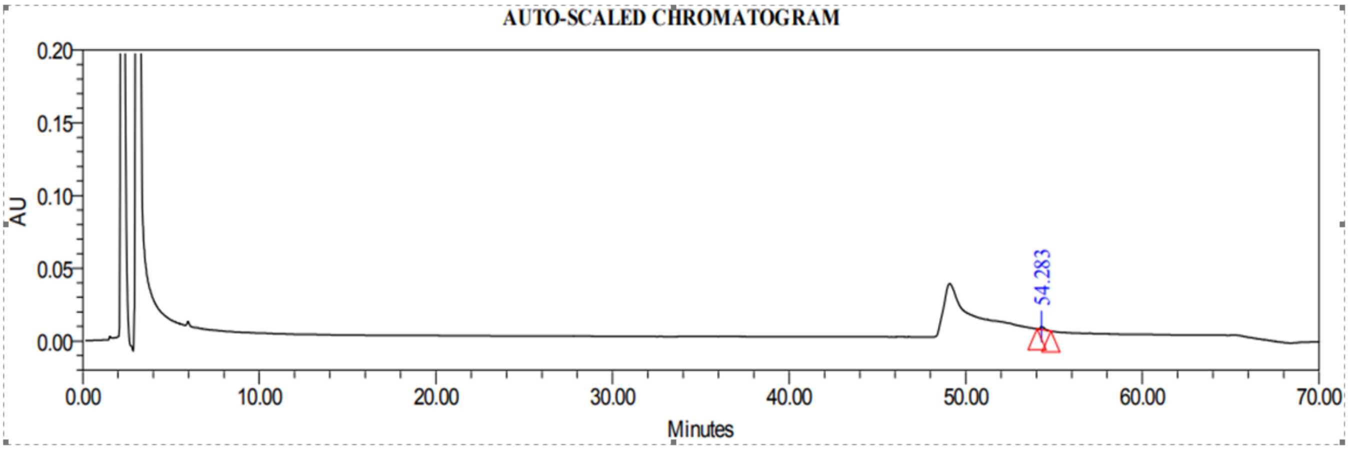

Figure 6 Typical Chromatogram of Placebo solution

3.6. Forced Degradation Study

For this analysis forced degradation studies are carried out with Acid hydrolysis, Base hydrolysis, Oxidation, Thermal stress, Humidity, Photolytic conditions revealed that the percentage of total degradation, indicative of major degradation impurities/products formed in relation to relative retention, Mass balance calculations, Peak purity

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assessments. The findings from the stress conditions are summarized in Table 6 & Table 7. Notably, significant degradation was observed under base and peroxide conditions. Figure 7 represents the chromatogram of the base and peroxide degradation.

Table 6 Forced Degradation Results Individual Impurity and Single Maximum Impurity

S.No	Name of Degradation	%Known Impurities						
		Imp-H	Imp-B	Imp-L	Imp-D	Imp-F	Imp-E	Single Max Imp.
1	Test As such	ND	0.8758	ND	ND	ND	0.0189	ND
2	Acid degradation	ND	0.9544	ND	ND	ND	0.4015	ND
3	Base Degradation	ND	0.8620	ND	4.8203	0.3061	0.0721	0.5201
4	Peroxide Degradation	4.7654	0.9629	ND	ND	ND	ND	ND
5	Water Degradation	ND	0.8734	ND	1.0762	ND	ND	ND
6	Thermal Degradation	ND	0.8245	0.0402	ND	0.2946	0.1516	0.2805
7	Photolytic degradation	ND	0.8914	ND	2.4222	ND	ND	ND
8	Humidity Degradation	ND	0.9269	ND	ND	ND	ND	ND

Table 7 Mass balance Results and peak Purity Results

Mode of degradation	%Assay of ERT	% Net degradation	%Mass balance (Between 95.0 to 105.0)	ERT (Limit PA<PT)	
				PA	PT
Control sample	101.4	-	–	0.515	2.552
Stressed with 1mL, 0.1N HCl for 5 minutes at bench top	98.3	2.8834	100.8	2.611	2.763
Stressed with 2mL, 0.1N NaOH for 5 minutes at bench top	97.3	5.6859	102.6	1.537	3.236
Stressed with 1mL, 3% Peroxide for 20 minutes at bench top	97.0	4.8336	101.4	0.753	2.256
Stressed with 2mL, water, for 5 hours at bench top	98.4	1.0549	99.1	0.330	2.240
Dry Heating at 105° C for 48 Hrs	103.1	0.6967	103.4	0.826	2.407
Photolytic (200 watt Hours/ Square meter and 1.2 million lux hours)	101.6	2.8834	101.1	0.677	1.875
Humidity (90% RH at 25°C for 7 Days)	100.2	0.0322	99.8	0.804	2.857

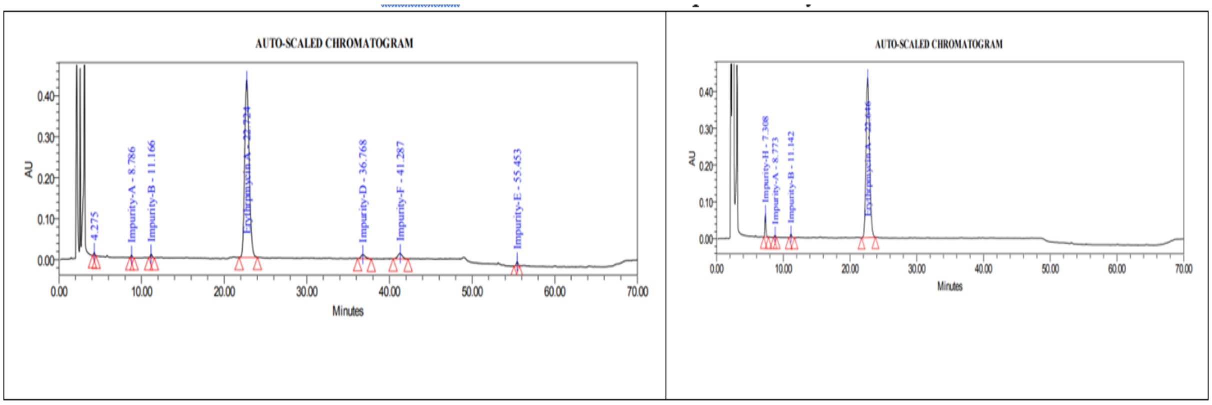

Figure 7 Typical Chromatogram of Base and Peroxide Degradation Sample

3.7. Robustness

These deliberate modifications were carried out to assess the impact on system suitability parameters and % recoveries, in comparison to the method's precision results. The results revealed that all these variations remained within a range of ± 10%. Consequently, the findings from these experiments demonstrate the robustness of the analytical method.

3.8. Stability of Mobile Phase and Sample Solutions

Stability assessments were conducted for the control sample solution, spiked sample solution, and dilute standard solutions used in Imp. determination, all under room temperature conditions. The evaluation of solution stability was carried out using freshly prepared standard solutions. The percentage recoveries were compared between the initial time (T) and time T + h hours. The results indicated that the sample solution remained stable for 1 hour at room temperature and 9 hours when stored in the refrigerator. Diluted standard solutions were found to be stable for 7 days at both room temperature and in the refrigerator. Similarly, the resolution solution exhibited stability for 7 days when stored in the refrigerator. Furthermore, the mobile phase remained stable for 7 days when kept at room temperature. The method provides selective quantification of ERT and related degradation products without interfering with blank and placebo, thereby affirming the method stability-indicating character.

Conclusion

The method for assessing related substances in ERT Tablets has undergone a comprehensive validation process, covering various critical parameters. The validation confirmed its effectiveness in terms of system suitability, specificity (interference from placebo and degradation products), linearity, method precision, ruggedness (intermediate precision), accuracy, establishment of LOD and LOQ, precision at LOQ, accuracy at LOQ, range (linearity, precision, and accuracy), stability of solutions (benchtop stability of standard and samples, as well as refrigerator stability of samples), mobile phase stability (on bench top), and robustness (variation in flow rate, column temperature, acetonitrile variation in Mobile phase-A, buffer variation in Mobile phase-A, buffer variation in Mobile phase-B, aqueous variation in Mobile phase-B, pH variation of buffer). This method was found to be linear from concentration range 3.6-182.8 for ERT-A,1.18-59.563 for IMP-H, 1.21-120.0 for IMP-B,0.23-24.69for IMP-L,4.67-58.47 for IMP-D, 0.48-62.51 for IMP-F, and 0.12-180.32 for IMP-E. This proposed method has demonstrated a high degree of specificity, selectivity, and robustness, making it exceptionally well-suited for the separation and quantification of impurities in ERT Tablets solution.

Conflicts of Interest

The authors declare that they have no conflicts of interest among the authors.

Ethical approval

This work did not contain any studies with human participants performed by any of the authors.

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